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The general mitochondrial processing peptidase from potato is an integral part of cytochrome *c* reductase of the respiratory chain

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The major mitochondrial processing activity removing presequences from nuclear encoded precursor proteins is present in the soluble fraction of fungal and mammalian mitochondria. We found that in potato, this activity resides in the inner mitochondrial membrane. Surprisingly, the proteolytic activity co-purifies with cytochrome *c* reductase, a protein complex of the respiratory chain. The purified complex is bifunctional, as it has the ability to transfer electrons from ubiquinol to cytochrome *c* and to cleave off the presequences of mitochondrial precursor proteins. In contrast to the nine subunit fungal complex, cytochrome *c* reductase from potato comprises 10 polypeptides. Protein sequencing of peptides from individual subunits and analysis of corresponding cDNA clones reveals that subunit III of cytochrome *c* reductase (51 kDa) represents the general mitochondrial processing peptidase.

Key words: cytochrome *c* reductase/mitochondria/potato/processing peptidase/protein import

Introduction

In fungi and mammals, and probably also in plants, most nuclear encoded precursors of mitochondrial proteins contain amino-terminal extensions that are important for mitochondrial targeting and intramitochondrial sorting (for review see Hurt and van Loon, 1986; Pfanner and Neupert, 1990). These so-called presequences are, in most cases, partly or completely cleaved off upon import into the organelle. To date, only a few plant mitochondrial presequences have been characterized in detail (Bowler *et al.*, 1989; White and Scandalios, 1989; Braun *et al.*, 1992). As fungal extension peptides have been shown to function as signals for targeting proteins to tobacco mitochondria (Schmitz and Lonsdale, 1989; Huang *et al.*, 1990), it is generally assumed that the protein import machinery of fungi and plants is evolutionarily conserved (Chaumont *et al.*, 1990). On the other hand, the import efficiency may be significantly reduced if a yeast presequence is used to target a passenger protein into tobacco mitochondria (Schmitz *et al.*, 1990). This raises the possibility that certain features of the mitochondrial translocation apparatus may differ between plants and fungi. We have chosen potato (*Solanum tuberosum* L.) as a model system to characterize components that interact with the presequences of mitochondrial precursor proteins during import.

Mitochondrial presequences are supposed to interact specifically with receptor proteins localized in the outer mitochondrial membrane (reviewed in Pfanner *et al.*, 1991) and with processing peptidases inside the mitochondria. At least two different processing activities mediating the partial or complete removal of mitochondrial presequences are present in the matrix of fungal and mammalian mitochondria. The commonly known proteolytic activity inside mitochondria is that of the matrix processing peptidase, which was first identified more than a decade ago (Böhni *et al.*, 1980). Recently the purification of this processing activity from *Neurospora* (Hawlitschek *et al.*, 1988) and yeast (Pollock *et al.*, 1988; Yang *et al.*, 1988) mitochondria revealed that two proteins are involved in the removal of matrix targeting signals. The catalytic polypeptide is the matrix processing peptidase (MPP, termed Mas2 in yeast, Yaffe and Schatz, 1984) which in *Neurospora* and yeast exists as a soluble component in the matrix space. For activity the presence of a so-called 'processing enhancing protein' (PEP, termed Mas1 in yeast) is required. The *Neurospora* PEP was shown to be identical with subunit I of cytochrome *c* reductase while, in yeast, PEP (Mas1) and subunit I are different proteins (Schulte *et al.*, 1989). As about a quarter of *Neurospora* PEP is found in the mitochondrial matrix, it has been suggested that the soluble PEP molecules are involved in the removal of mitochondrial presequences *in vivo* (Arretz *et al.*, 1991). In mammals, the processing enhancing function has not been demonstrated directly although a two component matrix protease has been isolated from rat liver mitochondria with one subunit being homologous to fungal MPP (Ou *et al.*, 1989; Kleiber *et al.*, 1990). The mammalian enzyme was denoted 'general matrix processing protease' or 'matrix protease I' to indicate the existence of another matrix endopeptidase. This protease, termed 'matrix protease II' or 'mitochondrial intermediate protease' (MIP) removes the second part of amino-terminal presequences of some precursor proteins, e.g. ornithine carbamoyl transferase (Kalousek *et al.*, 1988; Isaya *et al.*, 1991). In *Neurospora*, this enzyme has been shown to eliminate the carboxyl-terminal eight residues of the presequence of the Rieske-FeS protein (Hartl *et al.*, 1986). While MPP and MIP are components of the soluble fraction of mitochondria, another protease with homologies to *Escherichia coli* leader peptidase is located in the mitochondrial inner membrane (Baker and Schatz, 1991). This protein, termed 'inner membrane protease I', has been recently characterized in yeast (Behrens *et al.*, 1991; Schneider *et al.*, 1991). This protease acts on presequences of proteins located in the mitochondrial intermembrane space, e.g. cytochrome b_2 and the mitochondrially encoded subunit II of cytochrome oxidase. There is genetical evidence for the presence of another protease in the inner membrane acting on the presequences of cytochrome c_1 and cytochrome *c* peroxidase (Pratje and Guiard, 1986).

Here we report the purification of the first plant

mitochondrial processing peptidase and the molecular characterization of its catalytically active subunit. This protease is different from the yeast inner membrane protease and homologous to the general matrix processing peptidase. Unexpectedly, the plant endoprotease is not a soluble matrix protein as in fungi and mammals, but is entirely associated with the membrane fraction. We provide evidence that it forms an integral part of cytochrome *c* reductase of the respiratory chain.

Results

Submitochondrial localization of the processing peptidase: processing activity co-fractionates with the mitochondrial membranes

While in fungi the preparation of mitochondrial enzymes may start with total cell protein (Hawlitschek *et al.*, 1988), in plants the first purification step has to be the preparation of mitochondria as their proteins represent only a minor portion of total cell protein. Mitochondria were isolated from ~100 kg of potato tubers and the submitochondrial localization of the processing activity was determined prior to further purification steps. To ensure the purity of the subfractions, antibodies directed against specific markers of the matrix and membrane fraction were used (Figure 1A). An *in vitro* synthesized radiolabelled precursor of the β-subunit of the mitochondrial ATPase from tobacco was incubated with different lysates from potato tuber mitochondria (see Figures 1 and 4) . In accordance with the data of Whelan *et al.* (1988) the precursor protein corresponding to a band of 56 kDa is processed to the mature form (52 kDa). Upon subfractionation of the mitochondria only the membrane fraction, but not the matrix, retains the processing activity (Figure 1B and C). One microgram of membrane protein leads to processing of part of the precursor while 100 μg of matrix protein does not exhibit detectable processing activity. This suggests that potato mitochondrial membranes contain at least 100 times more processing activity than the soluble fraction.

The processing activity was further characterized with antibodies against the matrix protease from fungi. The antibodies specifically recognize potato mitochondrial proteins (not shown) and were therefore used for immunoprecipitation. As revealed by SDS−PAGE, the immunoprecipitate obtained from solubilized potato mitochondria contained a specific set of proteins. Unexpectedly, some of these polypeptides were identified as subunits of cytochrome *c* reductase (not shown). Thus we reasoned that applying a gentle procedure for the purification of cytochrome *c* reductase from potato mitochondria might lead to the isolation of the mitochondrial processing enzyme.

The membrane bound processing activity from potato mitochondria co-fractionates with cytochrome c reductase

Cytochrome *c* reductase was purified with the procedure developed by Weiss in the late seventies (H.-P.Braun and U.K.Schmitz, submitted). The main purification steps are a cytochrome *c* affinity column which takes advantage of the specific interaction between cytochrome c_1 and cytochrome *c* and a gel filtration column. A prerequisite for employing this method is the preparation of monodisperse cytochrome *c* reductase complexes using appropriate concentrations of a non-ionic detergent like Triton X-100 (Weiss and Juchs, 1978).

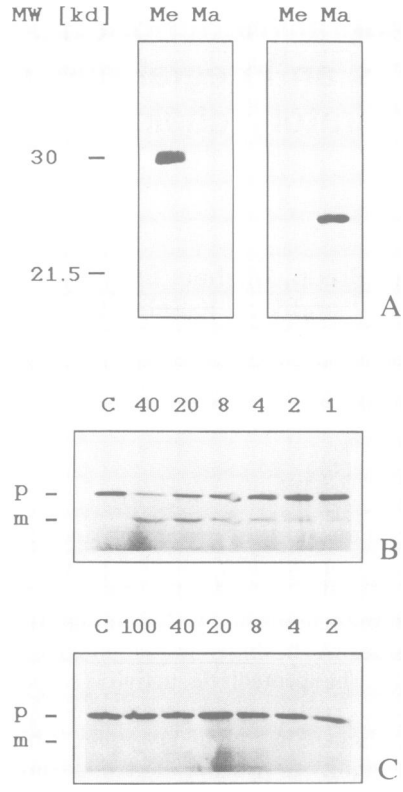

Fig. 1. Submitochondrial localization of the processing activity of potato mitochondria. Mitochondria were subfractionated into membrane (Me) and matrix (Ma) fractions by sonication and ultracentrifugation as described in Materials and methods. (**A**) Purity of subfractions of potato mitochondria as revealed by immunoblotting. The blot was incubated with antibodies against the ADP/ATP translocator (left two lanes), an integral protein of the inner membrane, and with manganese superoxide dismutase (right two lanes), a marker enzyme of the mitochondrial matrix. A 30 kDa protein representing the ADP/ATP translocator is exclusively present in the membrane fraction while a 25 kDa protein corresponding to the manganese superoxide dismutase only occurs in the matrix. Each lane contains ~20 μg protein. (**B**) Processing activity is located in the membrane fraction. Labelled precursor of the β-subunit of F_1-ATPase was incubated with mitochondrial membrane proteins and analysed by SDS−PAGE and fluorography. The numbers above the lanes indicate the amount of membrane protein (in μg) used in the assay. Part of the precursor protein (p) is processed to the mature form (m). As a control (C) Laemmli buffer was added to 40 μg of membrane proteins immediately after addition of the precursor. (**C**) No activity is detectable in the matrix fraction of potato mitochondria. The labelled precursor was incubated under the same conditions as in (B) with different amounts of matrix protein (indicated above the lanes).

Application of this procedure to potato mitochondria yields 10 highly pure polypeptides, most of which have molecular weights similar to the nine subunit cytochrome *c* reductase from *Neurospora* and yeast (Figure 2A). Their appearance in distinct fractions eluted from the affinity column and the gel filtration column correlates with the occurrence of absorption maxima for cytochromes *b* and c_1 (not shown). All fractions containing these bands exhibit ubiquinol cytochrome *c* oxidoreductase activity which can be inhibited by myxothiazol and antimycin (H.-P.Braun and U.K. Schmitz, submitted).

The identity of individual subunits was analysed by electro-immunoblotting. Antibodies directed against the five

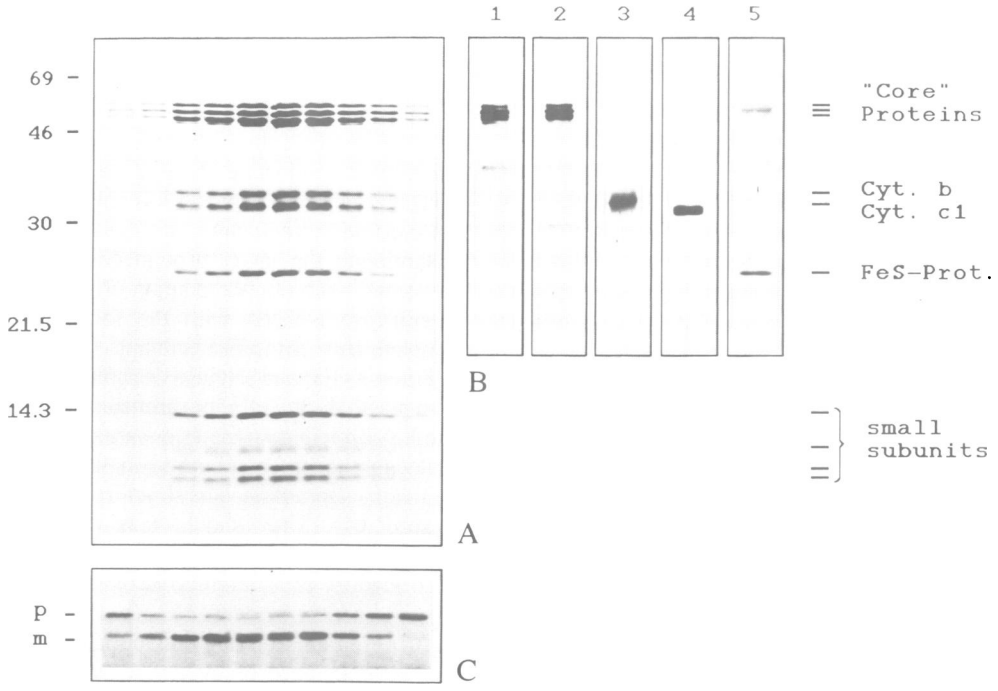

Fig. 2. Co-purification of cytochrome *c* reductase and the membrane bound processing activity. (**A**) Coomassie stained SDS−polyacrylamide gel of 10 successive fractions from a gel filtration column. Ten main polypeptides elute as a symmetric peak with a maximal protein concentration of 1 mg/ml. Their occurrence correlates with ubiquinol cytochrome *c* oxido-reductase activity (H.-P.Braun and U.K.Schmitz, submitted). (**B**) Identification of individual subunits of potato cytochrome *c* reductase. Immunoblots of cytochrome *c* reductase fractionated by SDS−PAGE after incubation with antibodies directed against subunits I and II of cytochrome *c* reductase from *Neurospora* (1 and 2), antibodies directed against cytochrome *b* and cytochrome c_1 from yeast (3 and 4) and an antibody directed against the iron sulfur protein from *Neurospora* (5). Each lane contains ~0.5 μg of protein. (**C**) Incubation of the radiolabelled precursor of the β-subunit of ATPase with equal volumes of the fractions shown in (A). The processing conditions were the same as in Figure 1.

largest subunits of yeast and *Neurospora* cytochrome *c* reductase reacted with protein bands of the six peak fractions from the last purification step (Figure 2B). Serum made against subunits I and II (the so-called 'core' proteins) of the bc_1 complex from *Neurospora* labels the 55, 53 and 51 kDa subunits of potato cytochrome *c* reductase. In both cases the strongest reaction is with the 53 kDa polypeptide. The cross-reaction with the other proteins is not surprising as, in fungi, subunits I and II belong to the same protein family. Antibodies directed against the redox centre containing subunits III−V (cytochrome *b*, cytochrome c_1 and the iron sulfur protein) of the fungal bc_1 complex cross-react specifically with individual polypeptides representing subunits IV−VI in potato. This indicates that one of the three largest subunits of potato cytochrome *c* reductase represents an additional polypeptide not present in the fungal protein complex.

The presence of an extra subunit in potato cytochrome *c* reductase and the finding that antibodies directed against fungal MPP co-immunoprecipitate subunits of this respiratory complex caused us to test whether the isolated complex exhibits processing activity towards mitochondrial precursor proteins. As shown in Figure 2C, purified cytochrome *c* reductase eluted from the gel filtration column processes the precursor of the β-subunit of mitochondrial F_1-ATPase to the mature form.

Properties of the three largest subunits of potato cytochrome c reductase

The additional polypeptide of the potato cytochrome *c* reductase preparation could potentially be a contamination,

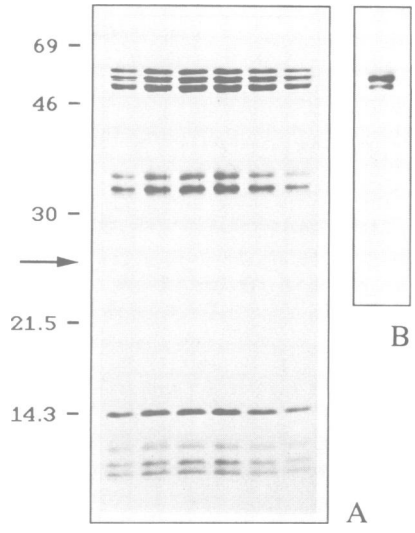

Fig. 3. Analysis of some properties of the three largest subunits of cytochrome *c* reductase. (**A**) The three high molecular weight polypeptides form an integral part of cytochrome *c* reductase. The protein complex was diluted in the presence of 0.04% Triton X-100 and 40 mM Tris−acetate (pH 7.0) during affinity chromatography and gel filtration and subsequently analysed by SDS−PAGE. A protein band of 25 kDa (arrow) representing the FeS protein (see Figure 2) is missing from the complex while all the other proteins of the complex, including the three largest subunits, are still present in unchanged stoichiometry. (**B**) Immunoblot of cytochrome *c* reductase fractionated by SDS−PAGE after incubation with an antibody directed against the Mas2 encoded processing peptidase from yeast. The 53 and 51 kDa polypeptides are recognized by the antibody.

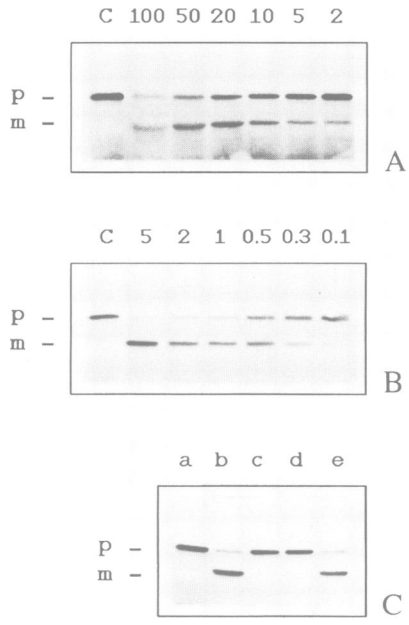

Fig. 4. Characterization of the processing activity of cytochrome *c* reductase. To establish the purification factor of the processing activity, defined amounts of total mitochondrial protein (**A**) or purified cytochrome *c* reductase (**B**) were incubated with the labelled precursor of the β-subunit of ATPase under the conditions described in Figure 1. About 50% of the precursor protein (p) is processed to the mature form (m) by 20 μg of total mitochondrial protein or 0.5 μg of cytochrome *c* reductase. (**C**) The radiolabelled precursor of the β-subunit of F_1-ATPase from tobacco (lane a) was incubated with 1 μg of purified cytochrome *c* reductase. Processing of the precursor to the mature sized protein (lane b) is inhibited upon preincubation of cytochrome *c* reductase with 1 mM (lane c) or 3 mM (lane d) EDTA for 10 min. Addition of 2 mM $MnCl_2$ (lane e) reconstitutes processing activity.

but several points strongly argue against this possibility: (i) overloaded SDS–polyacrylamide gels (not shown) confirm that the preparations are pure after the second chromatography step; (ii) the three largest subunits specifically elute not only from the affinity column but also from the gel filtration column together with the peak fractions of cytochrome *c* reductase. To find out whether one of the high molecular weight polypeptides is only loosely associated with cytochrome *c* reductase, we tested different conditions destabilizing the intact protein complex. As reported by Weiss and Kolb (1979), dilution of *Neurospora* cytochrome *c* reductase isolated with the same procedure leads to complete loss of the FeS subunit. Dilution in the presence of Triton X-100 and salt also destabilizes the protein complex from potato during affinity chromatography and gel filtration. The FeS protein dissociates quantitatively from the complex while all the other subunits, including the three largest, maintain a supramolecular structure in unchanged quantities (Figure 3A). They should thus be regarded as integral polypeptides of potato cytochrome *c* reductase.

To identify the subunit(s) responsible for the processing activity, potato cytochrome *c* reductase was analysed by electro-immunoblotting with antibodies directed against the Mas2-encoded processing peptidase from yeast. Subunits II and III of the complex, with apparent molecular weights of 53 and 51 kDa on SDS–polyacrylamide gels, cross-react with the antibody (Figure 3B).

Characterization of the processing activity of cytochrome c reductase from potato

Isolation of potato cytochrome *c* reductase by affinity chromatography yields a protein complex which is purified 38-fold over isolated mitochondria (H.-P.Braun and U.K. Schmitz, submitted). To estimate the purification factor for

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                                                                                cgctaaaaagagaaccttgatctctgttctggatcacctggctttcaaaaataaaacacccaaaagggagtttgatt   77

tttctataaaatttctcaaagattttgaaattgaaaatctcttttgaaagacagacgacaacagaaagggatctaaagaagcgggatgttagaga  172

gggggaaacgaaaatgtaaatacacacttaatattttaggtagcctcactgatttttttccccctacaaaatcgcttttctcatcttcccggagc  267

                                                                  M   Y   R   C   A   S   S   R   L   S   S   L   K   A   R   Q    16
tctactcagttctccgatcatcaccgtcaaa ATG TAC AGA TGC GCA TCG TCT CGC CTC AGC TCT CTT AAG GCA CGT CAA  346

 G   N   R   V   L   T   R   F   S   S   S   A   A   V   A   T   K   P   S   G   G   L   F   S    40
GGC AAC AGG GTC TTG ACA AGA TTT TCA AGT TCA GCT GCT GTT GCA ACC AAG CCA TCT GGG GGT CTT TTT AGT  418

 W   I   T   G   D   T   S   S   S   V   T   P   L   D   F   P   L   N   D   V   K   L   S   P    64
TGG ATA ACT GGT GAT ACG TCA AGT TCA GTG ACT CCC TTG GAT TTC CCC CTC AAT GAT GTT AAA CTC TCG CCA  490

 P   L   P   D   Y   V   E   P   A   K   T   Q   I   T   T   L   A   N   G   L   K   V   A   S    88
CCA TTA CCT GAT TAT GTA GAA CCT GCA AAG ACC CAG ATA ACG ACT CTC GCC AAT GGT CTC AAA GTG GCC TCT  562

 E   A   S   V   N   P   A   A   S   I   G   L   Y   V   D   C   G   S   I   Y   E   T   P   A   112
GAA GCA TCG GTG AAC CCT GCT GCC TCA ATT GGC CTC TAT GTT GAC TGT GGC TCT ATT TAT GAG ACA CCA GCT  634

 S   Y   G   A   T   H   L   L   E   R   M   A   F   K   S   T   L   N   R   S   H   L   R   I   136
TCA TAT GGA GCC ACA CAC CTC TTG GAA CGC ATG GCC TTC AAA AGC ACA TTA AAC CGG AGT CAC TTG CGT ATT  706

 V   R   E   I   E   A   I   G   G   N   V   T   A   S   A   S   R   E   H   M   I   Y   T   Y   160
GTA CGA GAA ATT GAA GCA ATT GGT GGT AAT GTA ACA GCT TCA GCC TCA CGA GAG CAT ATG ATC TAC ACT TAT  778

 D   A   L   K   T   Y   V   P   Q   M   V   E   M   L   A   D   C   V   R   N   P   A   F   L   184
GAT GCT TTG AAA ACT TAT GTA CCA CAA ATG GTG GAG ATG CTT GCT GAC TGT GTT AGA AAT CCT GCA TTC CTG  850

 D   W   E   V   K   E   Q   L   E   K   V   K   A   E   I   S   E   Y   S   K   N   P   Q   H   208
GAT TGG GAA GTT AAA GAA CAG CTT GAG AAG GTT AAA GCT GAG ATT AGC GAG TAC TCC AAA AAC CCT CAA CAC  922
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 L  L  L  E  A  V  H  S  A  G  Y  A  G  P  Y  G  N  S  L  M  A  T  E  A  232
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 T  I  N  R  L  N  S  T  V  L  E  E  F  V  A  E  N  Y  T  A  P  R  M  V  256
ACA ATA AAC AGG TTA AAC AGC ACA GTG CTG GAG GAG TTT GTA GCT GAG AAT TAT ACT GCT CCT CGG ATG GTT 1066

 L  A  A  S  G  V  E  H  E  E  F  L  K  V  A  E  P  L  L  S  D  L  P  K  280
CTT GCT GCA TCT GGT GTT GAA CAT GAA GAG TTC TTA AAA GTT GCA GAA CCT CTT CTG TCT GAT TTA CCT AAG 1138

 V  A  T  I  E  E  P  K  P  V  Y  V  G  G  D  Y  R  C  Q  A  D  A  E  M  304
GTG GCC ACC ATT GAA GAA CCT AAA CCT GTG TAT GTG GGA GGA GAT TAC CGC TGT CAA GCC GAT GCA GAG ATG 1210

 T  H  F  A  L  A  F  E  V  P  G  G  W  M  S  E  K  E  S  M  T  L  T  V  328
ACT CAT TTT GCT CTT GCC TTT GAA GTT CCC GGT GGC TGG ATG TCT GAA AAA GAA TCA ATG ACT TTA ACA GTT 1282

 L  Q  M  L  M  G  G  G  G  S  F  S  A  G  G  P  G  K  G  M  Y  S  R  L  352
CTT CAG ATG CTT ATG GGA GGA GGT GGA TCT TTC TCA GCT GGC GGT CCT GGA AAA GGG ATG TAC TCA AGA TTA 1354

 Y  L  R  V  L  N  Q  Y  P  Q  I  H  A  F  S  A  F  S  S  I  Y  N  N  T  376
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 G  L  F  G  I  Q  G  T  T  S  S  D  F  G  P  Q  A  V  D  V  A  V  K  E  400
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 L  I  A  V  A  N  P  S  E  V  D  Q  V  Q  L  N  R  A  K  Q  A  T  K  S  424
CTT ATT GCG GTA GCA AAC CCT AGT GAA GTT GAC CAA GTA CAG CTA AAC CGT GCT AAA CAG GCA ACA AAG TCT 1570

 A  I  L  M  N  L  E  S  R  M  V  A  S  E  D  I  G  R  Q  L  L  T  Y  G  448
GCC ATT CTG ATG AAC TTG GAA TCC CGG ATG GTT GCA TCA GAA GAT ATC GGC AGA CAA CTT TTG ACA TAT GGA 1642

 E  R  N  P  V  E  H  F  L  K  A  I  D  A  V  S  A  K  D  I  A  S  V  V  472
GAG AGG AAT CCA GTG GAG CAT TTC TTG AAA GCC ATT GAT GCA GTT TCA GCA AAA GAT ATT GCT TCC GTT GTG 1714

 Q  K  L  I  S  S  P  L  T  M  A  S  Y  G  D  V  L  S  L  P  S  Y  D  A  496
CAG AAG CTT ATT TCT TCT CCT CTG ACC ATG GCA TCC TAT GGA GAT GTT CTC TCC CTC CCA TCA TAC GAT GCA 1786

 V  S  S  R  F  R  S  K  *                                                  504
GTC AGC AGC AGG TTC CGT TCC AAA taaatcaattgtcaactacgaagtacacagcaggactatcatgctgttatttttttaggttttt 1873

agaaaatctttacgcgtgtggctgtaattttcggaaaataagaagggttacgagctatcgataaattcagctctgtcaaagctttgagataaaga 1968

gcatctgggcacatggtttttgtgtatacagtttgtaattcattcagctttctttgatatttgataacataatgtcatagggcattactgctgca 2063

gctggaagtcatcagatttctctagttaacaattt                                        2098
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Fig. 5. Nucleotide and deduced amino acid sequence of potato MPP. The DNA sequence of the insert of cDNA clone pM1 is shown. The predicted amino acid sequence of the longest open reading frame is indicated above the middle base of each codon using the single letter code. The amino acid sequences of two peptides generated with endoproteinase Lys-C from the 51 kDa protein of cytochrome *c* reductase are boxed. The sequence reported has been deposited in the EMBL data library under the accession number X66284.

the processing activity, two series of defined amounts of mitochondrial lysates (Figure 4A) and purified cytochrome *c* reductase (Figure 4B) were incubated under identical conditions with equal volumes of labelled mitochondrial precursor protein synthesized in a single translation reaction. About half of the precursor is processed to the mature form by 20 μg of mitochondrial protein and 0.5 μg of cytochrome *c* reductase. This indicates a 40-fold purification of the processing enzyme over mitochondrial lysates, corresponding well with the purification factor for cytochrome *c* reductase.

To characterize the processing activity further, the precursors of different mitochondrial proteins (manganese superoxide dismutase, mitochondrial hsp70, Cox IV-DHFR) were incubated with the isolated respiratory protein complex. They were all processed to their mature size (not shown). The processing activity is insensitive to PMSF, but sensitive to metal chelators like EDTA (Figure 4C). Addition of 1 mM EDTA completely inhibits the activity. When the EDTA-inhibited protease is subsequently treated with excess manganese chloride, enzyme activity is restored to normal levels. The processing activity can also be restored with zinc ions (not shown) indicating that the enzyme involved is a metalloprotease. These properties of the isolated cytochrome *c* reductase are identical with those of the processing activity in potato mitochondrial membranes and of the general matrix processing peptidase from fungi and mammals.

The 51 kDa protein of the potato cytochrome c reductase is the mitochondrial processing peptidase

To analyse the identity of the two polypeptides recognized by antibodies directed against the *Mas2*-encoded MPP from yeast, the 53 and 51 kDa bands were subjected to direct protein sequencing. All subunits of cytochrome *c* reductase were fractionated by SDS–PAGE, blotted onto PVDF

membranes and subjected to gas phase sequencing (Lottspeich, 1985). The amino-termini of the high molecular weight polypeptides were not blocked but, probably due to their size, the polypeptides were unstable during the sequencing procedure. Therefore ~600 pmol of each protein were digested with endoproteinase Lys-C and the resulting polypeptides were separated by HPLC. The amino acid sequences of fractions from the 51 and 53 kDa proteins were determined by Edman degradation. Sequences obtained from the 53 kDa protein exhibit similarity with PEP from *Neurospora* and Mas1 from yeast (M.Emmermann, H.-P.Braun and U.K.Schmitz, in preparation). Both peptides from the 51 kDa protein comprising a total of 31 amino acids were completely identical with two stretches of the amino acid sequence deduced from cDNA clones which most probably encode the mitochondrial processing peptidase from potato (see below).

Isolation of cDNA clones and sequence analysis of the mitochondrial processing peptidase from potato

In parallel to the biochemical purification of the processing peptidase from potato mitochondria, we had started to isolate cDNA clones from an expression library. As screening with heterologous antibodies was not successful, synthetic oligonucleotides derived from regions conserved between MPP from yeast and *Neurospora* were used. One of the five different oligonucleotide mixtures gave a specific signal upon hybridization with potato poly(A) RNA (not shown) and was chosen for screening the cDNA libraries. Several positively reacting clones, one of which also gave a positive signal upon immunoscreening, were isolated. Sequence analysis of clones with similar insert sizes around 2 kb revealed the presence of a single open reading frame (ORF) encoding a protein of 504 amino acids (Figure 5). The initiation codon of this ORF is the fourth ATG in the sequence, but the preceding three are all closely followed by in-frame stop codons. Alignment of the amino acid sequence encoded by the longest ORF with the matrix processing peptidase from yeast, *Neurospora* and rat (Figure 6) reveals 30–35% sequence identity. There is also significant similarity between potato MPP and fungal or mammalian sequences of PEP and the 'core' proteins of cytochrome *c* reductase, which is in line with earlier findings that all these proteins belong to the same protein family. An alignment of sequences of MPP, PEP and the 'core' proteins from fungi reveals ~25% sequence identity between *Neurospora* MPP and the PEPs from *Neurospora* and yeast (Schulte *et al.*, 1989). Also, potato MPP shows 20% identity with the Mas1-encoded PEP from yeast, 24% with PEP from *Neurospora* and 15 and 12% with the 'core' proteins I and II from yeast.

The sequence encoded by pMI shows striking structural features common to all characterized MPPs. There is a highly acidic region between Glu187 and Glu201. A similar clustering of negative charges is found in the corresponding segment of the fungal and rat MPP sequences. All these sequences, including the one from potato, have the potential to form an α-helix and are supposed to be able to bind positively charged amphiphilic presequences. Another potentially important region is the rather hydrophobic area between Gly334 and Tyr353 which is predicted to form a β-turn structure. It starts with four glycine residues and is identical in *Neurospora*, yeast and potato MPP. Although some cysteine residues are conserved between MPP from fungi and rat none is conserved in potato. This makes it unlikely that cysteine residues are directly involved in processing of mitochondrial precursor proteins.

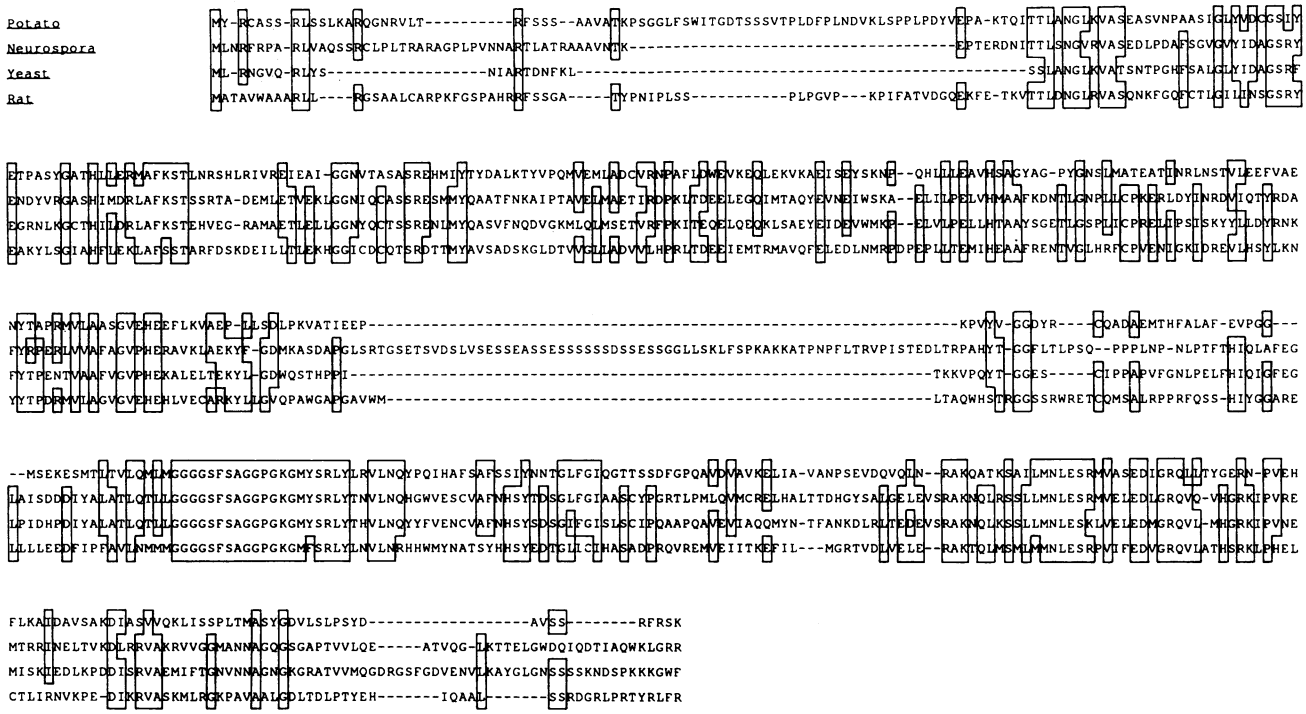

Fig. 6. Comparison of MPP from different organisms. Amino acid sequence alignment of mitochondrial processing peptidases from potato (this report), *Neurospora* (Hawlitschek *et al.*, 1988), yeast (Jensen and Yaffe, 1988) and rat (Kleiber *et al.*, 1990). Amino acids identical in at least three organisms are boxed.

Discussion

While in fungi and mammals the protein import apparatus has been investigated in great detail (for review see Pfanner and Neupert, 1990), little is known about proteins mediating the import of precursor proteins into plant organelles. None of the processing enzymes of plant mitochondria or chloroplasts have been purified to homogeneity or have been characterized at the molecular level. Here we report that, in plants, the enzyme homologous to the matrix processing peptidase from fungi and mammals is localized in the inner mitochondrial membrane and forms part of cytochrome *c* reductase, a protein complex of the respiratory chain. The purified complex exhibits strong processing activity upon incubation with different mitochondrial precursor proteins. Earlier investigations succeeded in preparing cytochrome *c* reductase activity from plant species but the number of its subunits was uncertain (Nakajima *et al.*, 1984; Degli Esposti *et al.*, 1985). In accordance with our data, a recent publication that appeared while this work was in progress describes for the first time the preparation of a plant cytochrome *c* reductase comprising 10 subunits and exhibiting high ubiquinol−cytochrome *c* oxidoreductase activity (Berry *et al.*, 1991). Three subunits with molecular weights of >50 kDa were found and discussed as allelic or tissue specific forms of one of the 'core' proteins. Neither in fungi (Siedow *et al.*, 1978; Weiss and Kolb, 1979) nor in mammals (Schägger *et al.*, 1986) does cytochrome *c* reductase contain more than two subunits with a molecular weight of >45 kDa. Of course it cannot be completely excluded that the occurrence of 10 subunits in cytochrome *c* reductase from potato is due to an artefact of the isolation procedure. However, for the following reasons this possibility is highly unlikely: (i) all biochemical criteria normally used to consider proteins as subunits of a respiratory chain complex have been fulfilled; (ii) different isolation protocols (Berry *et al.*, 1991; this report) yield the same number of subunits; (iii) the additional subunit occurs in the same stoichiometry as the other subunits of the complex. Our immunological experiments and the data obtained from direct protein sequencing and DNA sequencing of corresponding cDNA clones indicate that in potato, polypeptides homologous to both subunits of the soluble heterodimeric processing enzyme from fungi and mammals form part of cytochrome *c* reductase. Peptides obtained from subunit II (53 kDa) of the complex are similar to the processing enhancing protein from fungi (M. Emmermann, H.-P.Braun and U.K.Schmitz, in preparation). Peptides of subunit III (51 kDa) of cytochrome *c* reductase resemble MPP, the catalytically active subunit of the matrix processing peptidase. Apart from 30−35% amino acid identity with MPP from *Neurospora* (Schneider *et al.*, 1990), rat (Kleiber *et al.*, 1990) and the Mas2-encoded processing peptidase from yeast (Jensen and Yaffe, 1988; Pollock *et al.*, 1988) some striking structural similarities with these enzymes suggest that the protein encoded by corresponding cDNA clones really represents the general mitochondrial processing peptidase. Most notable is a highly conserved hydrophobic region within the otherwise hydrophilic protein which may be located in the interior of the molecule and be important for the processing function (Kleiber *et al.*, 1990; Schneider *et al.*, 1990). Another potentially important region is a rather acidic stretch of 15 amino acids (residues 187−201) which has the potential to form an α-helix. The equivalent segment in MPP from fungi and mammals has a similar charge and secondary structure prediction and has been proposed to be involved in the binding of presequences which typically form amphiphilic helices with a hydrophobic and a positively charged face (Heijne, 1986; Roise *et al.*, 1988; Kleiber *et al.*, 1990). As suggested by Rawlings and Barrett (1991), the mitochondrial processing peptidases may belong to a new superfamily of metalloendoproteases lacking the classical zinc atom binding motif His-Glu-Xxx-Xxx-His. Possible zinc binding motifs conserved between the members of this family are His108, His213 and Glu189 of human insulinase. These residues are also conserved in potato MPP (His118, His214 and Glu190).

The specific features of the protein encoded by clone pM1 argue against the possibility that it represents a polypeptide other than MPP, although it does have high sequence homology with PEP and subunit I of cytochrome *c* reductase from fungi. As recently reported by Schulte *et al.* (1989), all of these proteins belong to the same protein family which includes the two components of the mitochondrial processing peptidase, subunits I and II of cytochrome *c* reductase and a subunit of complex I of the respiratory chain (Röhlen *et al.*, 1991). These proteins either are located in the mitochondrial matrix (e.g. MPP and PEP from fungi) or face the matrix space forming part of respiratory chain complexes like the 40 kDa subunit of NADH dehydrogenase or subunit I of cytochrome reductase, which in *Neurospora* is identical with the processing enhancing protein. MPP from potato is the first plant protein belonging to this protein family and, in contrast to the situation in fungi and mammals, it is tightly bound to cytochrome *c* reductase. It adds another component to this protein complex which already has six more subunits than the simpler three-subunit bacterial complex (Gabellini, 1988; Trumpower, 1990). These additional subunits, which do not participate in electron transfer, may have evolved to perform certain functions resulting from the specific requirements that originated from the 'symbiotic' situation of the progenitor of mitochondria in the eukaryotic cell. It is possible that in the ancestor of recent eukaryotes the components of the processing enzyme were already part of complex III of the respiratory chain and that they became detached to perform specific functions as in yeast, where respiration is repressed under anaerobic conditions while protein import and processing still occur. Alternatively, the association of important functions like protein import and processing with a respiratory complex may be regarded as a progressive feature which might be important for regulatory processes. It has already been suggested that the two 'core' proteins of cytochrome *c* reductase or the 40 kDa subunit of NADH dehydrogenase 'might function as a control unit, sensing what is going on inside the mitochondria' (Weiss *et al.*, 1990; Röhlen *et al.*, 1991). We can speculate that the complete association of the processing enzyme from potato with cytochrome *c* reductase might be of functional significance for the control of respiratory activity and protein import. The mode of this control can now be approached experimentally.

Materials and methods

Isolation and subfractionation of potato mitochondria

Potatoes (var. 'Hansa') were purchased locally and mitochondria were isolated basically as described previously (Braun *et al.*, 1992). The procedure

includes differential centrifugation steps and fractionation on Percoll step gradients yielding highly pure potato mitochondria which are physiologically active. Mitochondria purified in this way were used for *in vitro* processing assays as well as for our first preparations of cytochrome *c* reductase. Since Percoll gradients are only effective if moderately loaded, less time consuming procedures were tested. A method replacing the gradient purification step by two simple differential centrifugations (5 min at 3000 *g* followed by 30 min at 18 000 *g*) was designed. Using a soft paint brush, the pellet from these centrifugations was divided into three parts corresponding to the fractions usually seen in Percoll step gradients: a white pellet consisting of amyloplasts, starch and high density particles, a brown fraction containing the mitochondria and a yellow fraction containing broken organelles, proteins and pigments. The brown fraction was resuspended and centrifuged for 10 min at 18 000 *g*. Cytochrome *c* reductase isolated from this fraction by cytochrome *c* affinity chromatography and gel filtration (see below) had the same polypeptide composition, spectral properties, etc. as the enzyme from Percoll purified mitochondria.

Mitochondria were subfractionated by sonication with a Sonifier B12 (Branson, Danbury, USA) for four intervals of 10 s. Unbroken mitochondria were pelleted by centrifugation at 5000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 150 000 *g* for 90 min. The resulting pellet contains mitochondrial membranes while the soluble fraction mainly represents the mitochondrial matrix. The purity of subfractions was analysed on immunoblots with antibodies directed against the matrix enzyme manganese superoxide dismutase (kindly supplied by C.Bowler, Gent) and the ADP/ATP translocator (kindly given by W.Neupert, München).

Purification of cytochrome c reductase

Cytochrome *c* reductase was isolated by cytochrome *c* affinity chromatography following the procedure developed by Weiss and Juchs (1978) for the preparation of the *Neurospora* enzyme. Some modifications were introduced for optimal yield and integrity of potato cytochrome *c* reductase. Mitochondrial membranes were prepared by sonicating as described above and resuspended in water. Membrane proteins were solubilized with Triton X-100 (final concentration: 3.3%) and briefly centrifuged (10 min, 60 000 *g*) to remove lipids and membrane fragments. The supernatant was applied to a cytochrome *c*–Sepharose column, which had been prepared by coupling cytochrome *c* from horse (Sigma) to CNBr activated Sepharose 4B (Pharmacia) as described by Weiss and Juchs (1978). The column was washed with buffer containing 20 mM Tris–acetate (pH 7.0), 0.04% Triton X-100, 5% sucrose and 0.2 mM PMSF. Proteins bound to the column were then eluted with a linear gradient of 20 to 200 mM Tris–acetate (pH 7.0) and 2 mM ascorbate in the washing solution. Fractions containing cytochrome *c* reductase were identified spectrophotometrically by determination of the cytochrome *b* content (H.-P.Braun and U.K.Schmitz, submitted). They were pooled, concentrated and passed through an Ultrogel AcA 34 gel filtration column in 40 mM Tris–acetate (pH 7.0), 0.04% Triton X-100 and 0.2 mM PMSF (Weiss and Kolb, 1979). The purified protein complex was stored at −70°C.

Analysis of proteins by SDS–PAGE, immunoblotting and gas phase sequencing

The protein concentrations of mitochondrial fractions were determined with the method of Bradford (1976) using a kit supplied by Bio-Rad. Polypeptides fractionated in SDS–polyacrylamide slab gels (Laemmli, 1970) were either stained with Coomassie Brilliant Blue R250 or blotted onto nitrocellulose (Schleicher & Schüll). Blots were incubated overnight with different antibodies directed against the processing peptidase and individual subunits of cytochrome *c* reductase from yeast and *Neurospora*. The antibodies were kindly provided by W.Neupert, München, G.Schatz, Basel and H.Weiss, Düsseldorf. Visualization of immunopositive bands was performed with biotinylated antibodies (Vector Laboratories), avidin and horseradish peroxidase as previously described (Braun *et al.*, 1992). For protein sequencing, polypeptides were fractionated in 12% polyacrylamide gels which had been allowed to polymerize overnight. After blotting onto Immobilon membranes, protein bands were stained with Ponceau S (Sigma), cut out and digested overnight with endoproteinase Lys-C as described elsewhere (Graack *et al.*, 1991). The resulting peptides were separated by HPLC and subjected to Edman degradation in an Applied Biosystems pulsed liquid phase sequencer (Lottspeich, 1985). Phenylthiohydantoin amino acids were separated on-line in an Applied Biosystems model 120A analyser and identified by manual interpretation of the data.

Synthesis of radiolabelled precursor proteins and in vitro processing

The cDNA of the β-subunit of tobacco ATPase (Boutry and Chua, 1985) was transcribed *in vitro* under the control of the T3 promoter using RNA polymerase buffer (BRL). The reaction mixture contained 1 μg template DNA, 100 U RNAsin (Promega) and 0.5 mM of the cap analogue m^7GpppG (Pharmacia). After 10 min at 37°C the GTP concentration was raised to 0.5 mM and the reaction was allowed to continue for another 30 min. It was stopped by a heat treatment (5 min at 70°C) and the mRNA was extracted with phenol–chloroform (1:1), precipitated with ethanol and stored at −70°C in sterile water. Translation was performed in the presence of [^{35}S]methionine with rabbit reticulocyte lysate (Amersham) according to the supplier's instructions. *In vitro* processing of radiolabelled precursor proteins was carried out in a final volume of 100 μl processing buffer containing 22 mM Tris–HCl (pH 8.0), 0.6% Triton, 25 mM NaCl, 0.1 mM $ZnCl_2$, 0.1 mM $MnCl_2$, 0.1 mM $MgCl_2$, 1 mM PMSF. The buffer included 100 μg total mitochondrial protein or 10 μg of subfractionated mitochondria or 1 μg of purified cytochrome *c* reductase and was supplemented with 1–2 μl translation assay. The processing reaction was carried out for 1 h at 28°C; it was stopped with an equal volume of Laemmli buffer (2-fold concentrated, Laemmli, 1970) and incubated for 10 min at 60°C prior to loading onto the gel. Processing products were separated on SDS–polyacrylamide gels; for fluorography, the gels were incubated for 30 min in Amplify (Amersham) and exposed to X-ray film at −70°C.

Screening of cDNA libraries

Poly(A) RNA from potato tubers and leaves was reverse transcribed and cloned into phage λgt11 as previously described (Emmermann *et al.*, 1991). Degenerate oligonucleotides derived from conserved regions of *Neurospora* and yeast MPP were synthesized. Five sets of oligonucleotide mixtures (between 17 and 26 nt long) containing the full complement of sequences that could potentially encode the conserved peptides were tested. Under various hybridization conditions only one oligonucleotide mixture containing 8192 different sequences specifically recognized a band on Northern blots of potato poly(A) RNA. This oligonucleotide was a 26mer with the following sequence: 5′-TACATNCCYTTNCCNGGNCCNCCNGC-3′ corresponding to the conserved peptide Ala-Gly-Gly-Pro-Gly-Lys-Gly-Met-Tyr. The oligonucleotides were end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham) according to Sambrook *et al.* (1989) and used for screening the cDNA library as described by Wallace and Miyada (1987).

Analysis of nucleic acids

DNA cloning and analysis were performed according to standard procedures (Sambrook *et al.*, 1989). The cDNA inserts of positively reacting plaques were cloned into Bluescript vectors (Stratagene) and sequenced on both strands following the method of Sanger *et al.* (1977). Computer analysis of DNA and protein sequences was done using the FASTA program (Lipman and Pearson, 1985) and programs of the PCGene package (IntelliGenetics).

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References

Arretz,M., Schneider,H. and Neupert,W. (1991) *Biomed. Biochem. Acta*, **50**, 403–412.

Baker,K.P. and Schatz,G. (1991) *Nature*, **349**, 205–208.

Behrens,M., Michaelis,G. and Pratje,E. (1991) *Mol. Gen. Genet.*, **228**, 167–176.

Berry,E.A., Huang,L.-S. and DeRose,V.J. (1991) *J. Biol. Chem.*, **266**, 9064–9077.

Böhni,P., Gasser,S., Leaver,C. and Schatz,G. (1980) In Kroon,A.M. and Saccone,C. (eds), *The Organisation and Expression of the Mitochondrial Genome*. Elsevier, Amsterdam, pp. 423–433.

Boutry,M. and Chua,N.-H. (1985) *EMBO J.*, **4**, 2159–2165.

Bowler,C., Alliotte,T., De Loose,M., van Montagu,M. and Inze,D. (1989) *EMBO J.*, **8**, 31–38.

Bradford,M. (1976) *Anal. Biochem.*, **72**, 248.

Braun,H.-P., Emmermann,M. and Schmitz,U.K. (1992) *Mol. Gen. Genet.*, **231**, 217–225.

Chaumont,F., O'Riordan,V. and Boutry,M. (1990) *J. Biol. Chem.*, **265**, 16856–16862.
Degli Esposti,M., Flamini,E. and Zannoni,D. (1985) *Plant Physiol.*, **77**, 758–764.
Emmermann,M., Braun,H.-P. and Schmitz,U.K. (1991) *Curr. Genet.*, **20**, 405–410.
Gabellini,N. (1988) *J. Bioenerg. Biomem.*, **20**, 59–83.
Graack,H.R., Grohmann,L. and Kitakawa,M. (1991) *Biochimie*, **73**, 837–844.
Hartl,F.-U., Schmidt,B., Wachter,E., Weiss,H. and Neupert,W. (1986) *Cell*, **47**, 939–951.
Hawlitschek,G., Schneider,H., Schmidt,B., Tropschug,M., Hartl,F.-U. and Neupert,W. (1988) *Cell*, **53**, 795–806.
Heijne, von G. (1986) *EMBO J.*, **5**, 1335–1342.
Huang,J., Hack,E., Thornburg,R.W. and Myers,A.M. (1990) *Plant Cell*, **2**, 1249–1260.
Hurt,E.C. and van Loon,A.P.G.M. (1986) *Trends Biochem. Sci.*, **11**, 204–207.
Isaya,G., Kalousek,F., Fenton,W.A. and Rosenberg,L.E. (1991) *J. Cell Biol.*, **113**, 65–76.
Jensen,R.E. and Yaffe,M.P. (1988) *EMBO J.*, **7**, 3863–3871.
Kalousek,F., Hendrick,J.P. and Rosenberg,L.E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7536–7540.
Kleiber,J., Kalousek,F., Swaroop,M. and Rosenberg,L.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7978–7982.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lipman,D. and Pearson,W. (1985) *Science*, **227**, 1435–1441.
Lottspeich,F. (1985) *Trends Anal. Chem.*, **4**, 244–246.
Nakajima,T., Maeshima,M. and Asahi,T. (1984) *Agric. Biol. Chem.*, **48**, 3019–3025.
Ou,W.-J., Ito,A., Okazaki,H. and Omura,T. (1989) *EMBO J.*, **8**, 2605–2612.
Pfanner,N. and Neupert,W. (1990) *Annu. Rev. Biochem.*, **59**, 331–353.
Pfanner,N., Söllner,T. and Neupert,W. (1991) *Trends Biochem. Sci.*, **16**, 63–67.
Pollock,R.A., Hartl,F.-U., Cheng,M.Y., Ostermann,J., Horwich,A. and Neupert,W. (1988) *EMBO J.*, **7**, 3493–3500.
Pratje,E. and Guiard,B. (1986) *EMBO J.*, **5**, 1313–1317.
Rawlings,N.D. and Barrett,A.J. (1991) *Biochem. J.*, **275**, 389–391.
Röhlen,D.A., Hoffmann,J., van der Pas,J.C., Preis,D., Sackmann,U. and Weiss,H. (1991) *FEBS Lett.*, **278**, 75–78.
Roise,D., Theiler,F., Horvath,S.J., Tomich,J.M., Richards,J.H., Allison,D.S. and Schatz,G. (1988) *EMBO J.*, **7**, 649–653.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5470.
Schägger,H., Link,T.A., Engel,W.D. and von Jagow,G. (1986) *Methods Enzymol.*, **126**, 224–237.
Schmitz,U.K. and Lonsdale,D.M. (1989) *Plant Cell*, **1**, 783–791.
Schmitz,U.K., Hodge,T.P., Walker,E. and Lonsdale,D.M. (1990) In Lamb,C. and Beachy,R. (eds), *Plant Gene Transfer*. Alan R.Liss Inc., New York, pp. 237–248.
Schneider,H., Arretz,M., Wachter,E. and Neupert,W. (1990) *J. Biol. Chem.*, **265**, 9881–9887.
Schneider,A., Behrens,M., Scherer,P., Pratje,E., Michaelis,G. and Schatz,G. (1991) *EMBO J.*, **10**, 247–254.
Schulte,U., Arretz,M., Schneider,H., Tropschug,M., Wachter,E., Neupert,W. and Weiss,H. (1989) *Nature*, **339**, 147–149.
Siedow,J.N., Power,S., De La Rosa,F.F. and Palmer,G. (1978) *J. Biol. Chem.*, **253**, 2392–2399.
Trumpower,B.L. (1990) *Microbiol. Rev.*, **54**, 101–129.
Wallace,R.B. and Miyada,C.G. (1987) *Methods Enzymol.*, **152**, 432–442.
Weiss,H. and Juchs,B. (1978) *Eur. J. Biochem.*, **88**, 17–28.
Weiss,H. and Kolb,H.J. (1979) *Eur. J. Biochem.*, **99**, 139–149.
Weiss,H., Leonard,K. and Neupert,W. (1990) *Trends Biochem. Sci.*, **15**, 178–180.
Whelan,J., Dolan,L. and Harmey,M.A. (1988) *FEBS Lett.*, **236**, 217–220.
White,J.A. and Scandalios,J.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3534–3538.
Yaffe,M.P. and Schatz,G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4819–4823.
Yang,M., Jensen,R.E., Yaffe,M.P., Oppliger,W. and Schatz,G. (1988) *EMBO J.*, **7**, 3857–3862.

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